PATENT
Attorney Docket No.: A57660-1/DJB

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	Examiner: UNKNOWN
Weiss et al.	Group Art Unit: 1804
Serial No. 08/270,4312	
Filed: July 5, 1994	
For: NOVEL GROWTH FACTOR RESPONSIVE PROGENITOR CELLS WHICH CAN BE PROLIFERATED IN VITRO	

DECLARATION UNDER M.P.E.P. §715.01(c)

Commissioner of Patents
and Trademarks
Washington, DC 20231

Sir:

The undersigned, Brent A. Reynolds hereby declares and states that:

1. I was a co-author of the abstract entitled "EGF- and TGFα-responsive striatal embryonic progenitor cells produce both neurons and astrocytes" printed in Soc. Neurosci. Abstr. Vol. 15 (Oct/Nov 1990) [hereinafter "the Abstract"]. Dr. Samuel Weiss and Dr. Wolfram Tetzlaff were also co-authors of the Abstract.

2. The subject matter disclosed in the Abstract is also described in the above-referenced patent application. Dr. Weiss and I are inventors of the subject matter claimed in the application, but Dr. Tetzlaff is not.

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3. At the time the Abstract was written, Dr. Tetzlaff was a faculty member at the University of Calgary. I had planned to begin my Ph.D. work in Dr. Tetzlaff's lab. Prior to the time that I was to work in Dr. Tetzlaff's lab, I was working on some experiments in Dr. Weiss' lab related to the subject matter of the Abstract. The project in Dr. Weiss' lab began to grow in size. I then changed my thesis project to the work that I was doing with Dr. Weiss.

4. Dr. Tetzlaff was listed as an author on the Abstract primarily because he financially supported me while the reported work was done. He also participated in discussions of the work. During these discussions with Dr. Weiss and me, Dr. Tetzlaff principally provided critique to the research findings. He also made suggestions as to certain cellular markers and histochemical techniques to use to identify of some of the differentiated cells in the cell cultures (specifically, the use of GFAP to identify astrocytes). The use of cellular markers to identify differentiated neural cells was well-known at the time.

5. Dr. Tetzlaff did not participate with Dr. Weiss and me in the design of the culture conditions and techniques that led to a method for long-term passaging of the undifferentiated cells which in turn led to the discovery that these cells were multipotent stem cells capable of self-renewal. Thus, because Dr. Tetzlaff did not participate in the discovery that multipotent stem cells can proliferate *in vitro*, he was not included as an inventor in the application.

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001 and that willful, false statements may jeopardize the validity/enforceability of the application or any patent issued thereon.

Dated: 25.8.94 Signature: 

Brent A. Reynolds, Ph.D.

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DECLARATION UNDER M.P.E.P. §715.01(c)

Commissioner of Patents
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Washington, DC 20231

Sir:

The undersigned, Samuel Weiss hereby declares and states that:

1. I was a co-author of the abstract entitled "EGF- and TGFα-responsive striatal embryonic progenitor cells produce both neurons and astrocytes" printed in Soc. Neurosci. Abstr. Vol. 15 (Oct/Nov 1990) [hereinafter "the Abstract"]. Dr. Dr. Brent A. Reynolds and Dr. Wolfram Tetzlaff were also co-authors of the Abstract.

2. The subject matter disclosed in the Abstract is also described in the above-referenced patent application which has a priority date of July 8, 1991.

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Dr. Reynolds and I are inventors of the subject matter claimed in the application, but Dr. Tetzlaff is not.

3. At the time the Abstract was written, Dr. Tetzlaff was a faculty member at the University of Calgary. His laboratory was adjacent to mine, but his area of research was different in that he was studying gene expression following axotomy and axon regeneration while I was studying neural cell cultures. Dr. Reynolds had planned to begin his Ph.D. work in Dr. Tetzlaff's lab. However, prior to the time that he was to work in Dr. Tetzlaff's lab, he was working on experiments in my lab related to the effects growth factors had on the survival of neural cells in culture. When we found proliferating cells in the neural cell cultures Dr. Reynolds and I designed experiments to determine whether the proliferating cells might be stem cells. At the time, no one else had reported the proliferation of neural stem cells in culture. Dr. Reynolds then changed his thesis project to the work being done in my lab.

4. Dr. Tetzlaff was listed as an author on the Abstract because he was Dr. Reynold's academic advisor and thus financially supported Dr. Reynolds at the time the Abstract was written. As is typically done amongst colleagues in a university setting, Dr. Tetzlaff took part in discussions and critique of experimental results coming out of my lab. However, as far as conceiving the idea and designing and executing the experiments that proved that neural stem cells could proliferate in culture, this is the work of Dr. Reynolds and me.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be

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true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001 and that willful, false statements may jeopardize the validity/enforceability of the application or any patent issued thereon.

Dated: 25 August, 1994 Signature: _____
Samuel Weiss, Ph.D.

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Attorney Docket No.: A57660-1/DJB

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DECLARATION UNDER 37 CFR 1.132

Commissioner of Patents
and Trademarks
Washington, DC 20231

Sir:

The undersigned, Brent A. Reynolds, hereby declares and states that:

1. I am a co-inventor of the subject application and I have read the arguments in the final Official Action (mailed February 2, 1994) to U.S. Ser. No. 07/726,812, of which the captioned-application is a continuation.

2. The Examiner states that Applicants "have failed to provide evidence showing proliferation of neural tissue from adults or juveniles". The Examiner also states that "Applicants have failed to provide evidence that cells derived from adult tissue actually give rise to multipotent stem cells and therefore that the mammalian neural tissue contained at least one multipotent stem cell".

3. Attached to this Declaration as Appendix A is the article "Generation of Neurons and Astrocytes from Isolated Cells of the Adult Mammalian Central

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Nervous System", which was published in March of 1992 in Science. The article describes the isolation and proliferation of adult neural stem cells using substantially the same techniques described in the examples of the specification. The experiments described in the article and the other experiments described below were performed either by me or by others under my supervision in the laboratories at Neurospheres Ltd., Calgary, Canada.

4. The Examiner states that "nestin, is found on undifferentiated neural tissue" but that Applicants "have failed to positively show for cells derived from adult tissues that undifferentiated cells have proliferated". On p. 1707 Appendix A, the dissociation of adult mice striata is described. The cells were plated in serum-free culture containing EGF. Most of the cells died (>98%), however the remaining cells started to undergo cell division, and formed a sphere of proliferating cells. As stated in the sentence bridging pages 1707 and 1708, virtually all the cells in these proliferating spheres were immunoreactive for nestin. This is evidence that undifferentiated neural cells obtained from adult tissue were proliferating.

5. The Examiner states that "Applicants have failed to provide evidence that cells derived from adult tissue actually give rise to multipotent stem cells and therefore that the mammalian neural tissue contained at least one multipotent stem cell". The sphere of cells which forms in the presence of EGF using the techniques described in Appendix A and in the specification are clonally-derived, i.e. they are the progeny of a single cell. On page 1708 of Appendix A, half way down the page in the second column, the induced differentiation of these clonally-derived cells is described. A single sphere was transferred onto poly-L-ornithine-coated glass cover slips. The cells continued to proliferate and eventually migrated from the sphere. Using dual-antigen indirect immunocytochemistry, it was determined that some of these cells were

immunoreactive for neuron-specific enolase (NSE) and some cells were immunoreactive for glial fibrillary acidic protein (GFAP), markers for neurons and glial cells, respectively. The remainder of the cells were immunoreactive for nestin, a marker for an undifferentiated neural cell. Thus, the cells of the clonally-derived sphere differentiated into glial or neuronal cells. These results support the claim that a multipotent neural stem cell, the progeny of which are capable of differentiating into neurons or glia, can be obtained from adult tissue and induced to proliferate and differentiate *in vitro*.

6. Regarding claims 18 and 20, the Examiner states that "Applicants have failed to provide evidence that adult tissues obtained from the adult mouse and human would give rise to multipotent stem cells and that the stem cells could be proliferated *in vitro* since Applicants have failed to test for the undifferentiated state (nestin positive)". The neural cells that were proliferated and differentiated in the experiments described above and in Appendix A were derived from adult mouse neural tissue. Thus, Applicants have demonstrated that adult tissues obtained from mice give rise to multipotent stem cells that are nestin positive and that proliferate *in vitro*.

7. Clonally-derived neurospheres have also been obtained from adult human tissue. During a routine biopsy, normal tissue was obtained from a 65 year old female patient. The biopsy site was the right frontal lobe, 6 mm from the tip of the frontal/anterior horn of the lateral ventricle. The tissue was prepared using substantially the same procedure outlined in Example 2 of the specification and cultured in a serum-free medium comprising DMEM/F12 with EGF and bFGF (20 ng/ml of each growth hormone), in T25 flasks (Nunclon). The flasks were examined every 2-3 days for neurosphere formation. Clonally-derived cells were passaged using single sphere dissociation: single neurospheres were triturated 100x in sterile aliquot tubes containing 200 µl of

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the media/hormone/EGF-bFGF solution before culturing in 24- or 96-well plates. First-passage neurospheres were plated on poly-ornithine and laminin coated coverslips and allowed to plate down for 14 days in media/hormone/EGF + bFGF. Some first passage neurospheres were plated on laminin (20 µg/ml) and poly-ornithine coated coverslips in media/hormone mix for 19 hours, then processed for nestin staining as outlined in Example 7 of the specification. Nestin staining indicated that the neurospheres, prior to the induction of differentiation (as described below) were nestin positive, indicative of the presence of immature undifferentiated cells.

8. Pass one human neurospheres were plated on a laminin coated substrate (see above). After 14 days, the cultures received a media change to media/hormone mix plus 1% fetal bovine serum and were allowed to differentiate for 7 days. Immunocytochemical analysis was then performed to determine different neural phenotypes. The differentiated cells were fixed with 4% paraformaldehyde in PBS for 20 minutes. The coverslips were washed three times (five minutes each) in PBS. For triple label immunocytochemistry, the cells were permeabilized for 5 minutes in .3% Triton-X in PBS followed by 2 washes with PBS. A first set of primary antibodies, MAP-2 (mouse monoclonal, 1:1000, Boehringer Mannheim) and GFAP (Rabbit polyclonal, 1:300, BTI), used to determine the presence of neurons and astrocytes respectively, were mixed in 10% normal goat serum in PBS. The cells were incubated at 37° C for 2 hours and then washed 3 times in PBS. A first set of secondary antibodies, goat anti-mouse rhodamine (Jackson Immuno Research) and goat anti-rabbit FITC (IgG, 1:100 Jackson Immuno Research) were mixed in PBS. The cells were incubated for 30 minutes at 37° C and then rinsed three times with PBS. The second primary antibody, O4 (mouse monoclonal IgM, 1:10) for oligodendrocytes, was mixed in 10% normal goat serum in PBS. The

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cells were incubated for 2 hours at 37° C. The second set of secondary goat anti-mouse AMCA IgM (1:100 Jackson Immuno Research) was mixed in PBS and cells were incubated for 30 minutes at 37°C. The cells were then rinsed twice in PBS and then in double distilled water before mounting with Fluorosave.

9. Attached to this Declaration as Appendix B (sheets 1-6) are photographs of the cells derived from adult human neural tissue which were proliferated and differentiated *in vitro*. Photograph A shows the neurospheres that were generated in the presence of EGF and bFGF. Nestin staining indicated that these were immature, undifferentiated cells. Photograph B shows the multiple neurospheres which were generated from a single first-pass neurosphere. On average, a single neurosphere will give rise to 17 new neurospheres per well in a 96-well plate. Differentiation of the neurospheres resulted in the expression of all three types of neural cells, i.e. neurons (Photograph E), astrocytes (Photograph F) and oligodendrocytes (Photograph D).

10. Regarding claim 87, the Examiner states that "Applicants have failed to disclose evidence that amphiregulin would have the claimed results". Cells were isolated from the striatum of a 14 day old mouse embryo using the technique outlined in Example 1 of the specification. Media (DMEM F12/10% hormone mix, see Example 4 of specification) was prepared. Amphiregulin was added to the media to a final concentration of 100 ng/ml. Cells were added to the growth factor containing media to give a final density of 200,000 cells/ml. The cells were grown in T75 flasks (Nunclon) and incubated at 37° C with a 5% CO_2 atmosphere. Clonally-derived neurospheres started to appear from day 5 onwards. The cells of the neurospheres stain positive for nestin. Attached as Appendix C is a 100x magnification of amphiregulin generated neurospheres (16 days old).

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11. Regarding claim 88, the Examiner states that "there is no evidence in the art that stem cells of any type may be proliferated without limit". A defining characteristic of a stem cell is that it is capable of self-maintenance, i.e. it can divide without limit (see the text from Molecular Biology of the Cell attached to the concurrently filed Preliminary Amendment as Exhibit A; see also Exhibit B of the Preliminary Amendment which is an article by Potten and Loeffler which discusses the characteristics of stem cells and Exhibit C of the Preliminary Amendment which is an article by Hall & Watt which sets forth the definition of a stem cell). Each division of a stem cell results in the formation of a daughter stem cell (as opposed to two committed progenitor cells). The cells cultured using the methods described in the application exhibit the characteristics of stem cells: they have the capacity for proliferation, self-maintenance and the production of a large number of differentiated progeny. The culturing and passaging of the neurospheres as described in the specification at Example 5 to line 17 of Example 6 has been repeated over 30 times over the course of 8 months with a arithmetic increase in the total number of viable undifferentiated cells. After about 6-7 days *in vitro*, a typical neurosphere will have several hundred cells, depending on the growth factors used. Following the dissociation of the neurospheres into single cells and their replating, the committed progenitor cells attach to the substrate and eventually differentiate. The stem cells, however, continue to proliferate and form new floating spheres. Thus, the claimed methods provide for the continual proliferation of stem cells but not of progenitor cells. While we have not calculated the total number of cells produced from a single multipotent CNS stem cell using these methods, we estimate that after 30 DIV, greater than 0.5 million cells are generated. Attached as Appendix D is a bar graph that exhibits the theoretical number of cells that can be generated using these methods.

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12. The methods described in the references cited by the Examiner focus on cells that adhere to the tissue culture substrate. Based on the description of these cells in the references, and from my research, the cells being described in these references are committed progenitor cells that have only limited capacity for proliferation prior to their differentiation. The multipotent stem cell that is proliferated using the methods claimed in the application is a relatively rare cell and represents less than 0.1% of the total cells isolated from the striatum of embryonic mice (day 14). In response to a growth factor such as EGF, these cells begin to proliferate after about 4 days *in vitro* (DIV) to form a small cluster of cells which continue to grow until, at about 7 DIV they lift off the substrate and float in suspension. The floating spheres can be easily removed and the perpetuation procedure reinitiated.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001 and that willful, false statements may jeopardize the validity/enforceability of the application or any patent issued thereon.

Dated: 25-8-94 Signature: B=R

Brent A. Reynolds